Cancer Science
Japanese Cancer Association JCA
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Combined adjuvants of poly(I:C) plus LAG-3-Ig improve antitumor effects of tumor-specific T cells, preventing their exhaustion

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Key words

Adjuvants, cancer vaccine, LAG-3, poly(I:C), T cell exhaustion

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Funding Information

NEC Corporation; Yamaguchi University.

Received October 8, 2015; Revised December 3, 2015; Accepted December 13, 2015

Cancer Sci **107** (2016) 398–406

doi: 10.1111/cas.12861

Therapeutic cancer vaccines are designed to treat cancer by boosting the endogenous immune system to fight against the cancer. In the development of clinically effective cancer vaccines, one of the most practical objectives is to identify adjuvants that are capable of optimizing the vaccine effects. In this study, we explored the potential of polyinosinic–polycytidylic acid (poly(I:C)) and LAG-3-Ig (soluble recombinant protein of lymphocyte activation gene-3 [LAG-3] extracellular domain fused with human IgG Fc region) as adjuvants for P1A tumor antigen peptide vaccine in a pre-established P815 mouse tumor model with a transfer of tumor-specific T cells. Whereas the use of poly(I:C) or LAG-3-Ig as a signal adjuvant induced a slight enhancement of P1A vaccine effects compared to incomplete Freund's adjuvant, combined treatment with poly(I:C) plus LAG-3-Ig remarkably potentiated antitumor effects, leading to complete rejection of pre-established tumor and long-term survival of mice. The potent adjuvant effects of poly(I:C) plus LAG-3-Ig were associated with an enhanced infiltration of T cells in the tumor tissues, and an increased proliferation and Th1-type cytokine production of tumor-reactive T cells. Importantly, the combined adjuvant of poly(I:C) plus LAG-3-Ig downregulated expressions of PD-1, LAG-3, and TIGIT on P1A-specific T cells, indicating prevention of T cell exhaustion. Taken together, the results of the current study show that the combined adjuvants of poly(I:C) plus LAG-3-Ig with tumor peptide vaccine induce profound antitumor effects by activating tumor-specific T cells.

Therapeutic cancer vaccine is a strategy to stimulate endogenous immune responses against tumor cells, with the aim of curing established cancers. This approach has been strengthened by the identification of tumor-associated antigens (TAA) in the 1990s, which enabled us to take advantage of short peptides of MHC class I/II epitopes, long peptides spanning multiple epitopes, or TAA protein itself, as Ag for vaccine.[1–3] Accordingly, a large number of clinical trials of cancer vaccine have been examined in various types of cancers worldwide.[1,4,5] Contrary to great expectations and enthusiasm, the vast majority of late-stage clinical trials of cancer vaccines resulted in a failure to meet primary or secondary endpoints of showing significant improvement in overall survival or progression-free/disease-free survival.[5–7] The only exceptional case so far is sipuleucel-T, a cell-based vaccine for patients with metastatic hormone-refractory prostate cancer, which was approved by the FDA in 2010.[8] Insufficient clinical efficacy in the majority of therapeutic cancer vaccines could be attributed to two potential reasons. First, TAA-specific T cells are rendered unresponsive due to exhaustion or anergy in patients with advanced cancer, being an obstacle to elicit productive immune responses against tumor cells.[6,9] Second, even TAA-specific T cells that are activated in lymphoid organs will be rendered deactivated when exposed to various immunosuppressive mechanisms in the tumor microenvironment.[6,10] The approaches of therapeutic cancer vaccines need to be improved to overcome these issues.

A potential strategy to address these problems in cancer vaccines is an optimization of adjuvants. An adjuvant is any substance used as a vaccine component that boosts immune responses against Ag. Various adjuvants including montanide (IFA), QS21 (saponin), monophosphoryl lipid A, liposomes, and cytokines, have been used in clinical trials of cancer vaccine.[1,4,5] Biological effects of adjuvants can be categorized into two major modes of action: an improved delivery of TAA and an acceleration of antitumor immune responses. The former effect is based on stabilization of vaccine formulations which leads to an extended presence and preferred biodistribution of vaccinated TAA *in vivo*. The latter effect is predominantly mediated by a stimulation of innate immune cells through signaling into pattern recognition receptors, particularly TLR, which leads to indirect activation of TAA-specific T cell responses. In spite of these potentials, the current formulations of adjuvant need to be further improved, so as to render therapeutic cancer vaccine capable of achieving sufficient clinical outcomes.

In this study, we investigated combination of poly(I:C) plus LAG-3-Ig as a novel adjuvant for therapeutic tumor peptide vaccine. Poly(I:C), a synthetic double-stranded RNA, binds TLR3, melanoma differentiation-associated protein 5, and retinoic acid-inducible gene I, induces production of type I IFN and IL-12, and upregulates cross-priming of DC, thus leading to activation of TAA-specific T cells.[11,12] LAG-3-Ig, a soluble recombinant protein of LAG-3 extracellular domain fused with human IgG Fc region, has been reported to competitively attenuate the LAG-3 inhibitory signal in T cells as well as to stimulate DC and monocytes by interaction with MHC class II molecules.[13–15] Whereas previous reports have indicated the potential of poly(I:C) and LAG-3-Ig as separate adjuvants for cancer vaccines,[11,16–21] no studies have investigated whether the combination of these two reagents could strengthen their effects. We explore poly(I:C) plus LAG-3-Ig as a novel combination of adjuvants that can synergistically enhance the therapeutic efficacy of cancer vaccine to stimulate tumor-specific T cell responses.

Materials and Methods

Mice and cell lines. In all experiments, 6–10-week-old female DBA/2 mice, purchased from Japan SLC (Shizuoka, Japan), were used. P1A-specific TCR-transgenic mice[22] were originally generated and kindly provided by Dr. Yang Liu (The Children's Research Institute, Washington DC, USA), and backcrossed with DBA/2 mice at least 10 generations in our animal facility. All mice were maintained under specific pathogen-free conditions in the animal facility at Yamaguchi University (Ube, Japan). All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

P815 mastocytoma and L1210 lymphocytic leukemia, both syngeneic to DBA/2 mice, were purchased from ATCC (Manassas, VA, USA) and maintained *in vitro* with RPMI-1640 culture medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Gemini Bio Products, West Sacramento, CA, USA), 1% penicillin–streptomycin (Wako, Osaka, Japan), 25 mM HEPES, and 50 mM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, USA).

***In vivo* therapeutic model of pre-established tumor.** DBA/2 mice were inoculated s.c. with 5×10^5 P815 tumor cells in the lateral flank on day 0. On day 7, spleen cells from P1A-specific TCR-transgenic mice that contained 2×10^5 P1A-specific T cells identified as Vα8.3-positive cells by flow cytometry analysis were transferred i.v. into the mice. On days 8 and 15, the mice were injected s.c. with 50 μg P1A peptide (LPYLGWLVF; Sigma-Aldrich, St. Louis, MO, USA) mixed with the following adjuvants: 50 μL IFA (Sigma-Aldrich), 50 μg poly(I:C) (InvivoGen, San Diego, CA, USA), 1 μg LAG-3-Ig (Adipogen, San Diego, CA, USA), or 50 μg poly(I:C) plus 1 μg LAG-3-Ig. Tumor growth was measured periodically with digital calipers and tumor volume was calculated by the formula: tumor volume (mm^3) = (short diameter)^2 × long diameter / 2. Survival of the mice was also observed.

Those mice that had completely rejected tumor and survived over 100 days following treatment with P1A peptide vaccine mixed with adjuvants were rechallenged s.c. with 5×10^5 P815 cells in the left lateral flank and 5×10^5 L1210 cells in the right lateral flank. As a control, naïve DBA/2 mice were inoculated s.c. with P815 and L1210 by the same method. Tumor growth and survival of mice were monitored as above.

Histopathological and immunofluorescence analysis of tumor tissue. DBA/2 mice were inoculated with P815 tumor on day 0, injected with P1A-specific T cells on day 7, and then treated with P1A peptide vaccine with adjuvants on day 8, as described above. On day 14, tumors were excised from the mice and divided into two pieces by razor blade. One piece was immersed and fixed in 10% formalin solution, and used for H&E staining carried out by Biopathology Institute Co. Ltd (Oita, Japan). The other piece was embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) to generate frozen sections of tumor.

Immunofluorescence staining was carried out by using 5-μm thick sections cut from the frozen tumor tissue. Tissue sections were placed on a slide and fixed with methanol at −20°C for 10 min. The slides were then washed with PBS, followed by blocking with 3% BSA in PBS at room temperature for 30 min. Tissue sections were stained with anti-mouse CD4 Ab (rat IgG2b) and anti-mouse CD8α Ab (rat IgG2a) at 4°C overnight (both Ab were purchased from eBioscience, San Diego, CA, USA). The slides were then washed with PBS, followed by staining with Alexa Fluor 488-conjugated mouse anti-rat IgG2a Ab and Alexa Fluor 647-conjugated mouse anti-rat IgG2b Ab at room temperature for 60 min (both Ab were purchased from Abcam, Cambridge, MA, USA). Finally, the slides were washed with PBS and mounted with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). Observation of the slides was carried out using the BZ-X710 fluorescent microscope (Keyence, Osaka, Japan).

Cell proliferation and cytokine assay. DBA/2 mice were inoculated with P815 tumor on day 0, injected with P1A-specific T cells on day 7, and then treated with P1A peptide vaccine with adjuvants on days 8 and 15, as described above. On day 21, tumor-draining inguinal and axillary LNs were harvested and processed to single cell suspension. Lymph node cells (1.5×10^5 cells/well) were cocultured with 100-Gy irradiated P815 tumor cells (4×10^4 cells/well) in tissue-culture 96-well flat-bottom plates (Thermo Fisher Scientific). Proliferative activity of the cells was assessed by ^{3}H-thymidine incorporation during the last 10 h of 3 days of culture. Determination of the incorporated radioactive counts was measured by a TopCount NXT (PerkinElmer, Waltham, MA, USA).

To assess a cytokine production from tumor-reactive T cells, supernatants from the above coculture of tumor-draining LN cells and irradiated P815 cells were harvested after 3 days. The concentrations of various cytokines were measured by Bio-Plex Pro Mouse 23-plex assay kits according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry. DBA/2 mice were inoculated with P815 tumor and treated with P1A-specific T cell transfer and P1A peptide vaccine with adjuvants, as described above. On day 21, tumor-draining LN cells were harvested stained with BV421-conjugated anti-CD4 Ab, APC-conjugated anti-CD8 Ab, and FITC-conjugated anti-Vα8.3 Ab. To assess T cell exhaustion markers, the cells were further stained with PE-conjugated anti-PD-1 Ab, PE-conjugated anti-LAG-3 Ab, PE-conjugated anti-TIGIT Ab, and PE-conjugated anti-BTLA Ab. Flow cytometric data were acquired by the BD LSRFortessa X-20 cell analyzer (BD Biosciences, San Jose, CA, USA), and the data were analyzed by FlowJo Cytometry Analysis (Tree Star, Ashland, OR, USA). Antibodies used for FACS analysis were purchased from eBioscience or BioLegend (San Diego, CA, USA).

Statistical analysis. Unpaired, two-tailed Student's *t*-test was used for parametric data such as cytokine and proliferation

data; the log–rank test was used for mouse survival data. The results are expressed as the mean ± SD. Differences were considered to be significant at *P*-values less than 0.05.

Results

Eradication of pre-established P815 tumor by P1A peptide vaccine with combined adjuvant poly(I:C) plus LAG-3-Ig. In order to evaluate the efficacy of poly(I:C) and LAG-3-Ig as immunological adjuvants in tumor vaccine, we used an *in vivo* murine tumor model of pre-established P815 mastocytoma. DBA/2 mice, syngeneic to P815 tumor, were inoculated with P815 tumor cells s.c. on day 0. After 7 days, when the tumor mass reached approximately 6 mm in diameter, the mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly (I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. Although P1A is a dominant TAA in P815 tumor,[23] it has been reported that vaccination of P1A peptide alone is insufficient to induce regression of pre-established P815 tumor.[24] When P1A peptide vaccine with IFA adjuvant was given, all the mice suffered from outgrowth of tumor and died by day 50 (Fig. 1). Although vaccination of P1A peptide together with poly(I:C) or LAG-3-Ig adjuvant delayed the growth of P815 tumor, almost all the mice were eventually killed by the tumor. In sharp contrast, when the mice were treated with P1A peptide vaccine together with both poly(I:C) and LAG-3-Ig, pre-established P815 tumor completely regressed and all of the mice survived indefinitely. These results indicate that the combination of poly(I:C) plus LAG-3-Ig works as a highly potent adjuvant that enhances the antitumor therapeutic effects of P1A peptide vaccine.

Increased infiltration of T lymphocytes in tumor tissue by P1A peptide vaccine with poly(I:C) plus LAG-3-Ig adjuvant. We also carried out histopathological examinations of tumor tissues in mice treated with P1A peptide vaccine together with adjuvants. The mice were inoculated s.c. with P815 tumor on day 0, subsequently injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumor tissues were harvested and subjected to H&E staining as well as immunohistochemical staining of CD4 and CD8 to evaluate T cell infiltration. In the tissues from the mice treated with IFA as an adjuvant, massive growth of tumor cells with a lack of T cell infiltration was observed (Fig. 2). In the mice treated with either poly(I:C) or LAG-3-Ig, necrotic areas were observed in parts of tumor tissues, whereas only a slight infiltration of T cells was detected. In the mice treated with combined adjuvant of poly(I:C) and LAG-3-Ig, the majority of tumor tissues underwent necrotic changes, and an evident infiltration of CD4- and CD8-positive T cells adjacent to the necrotic area was observed. In addition, enhanced expressions of MHC class I and class II molecules were observed in tumor tissues from the mice treated with both poly

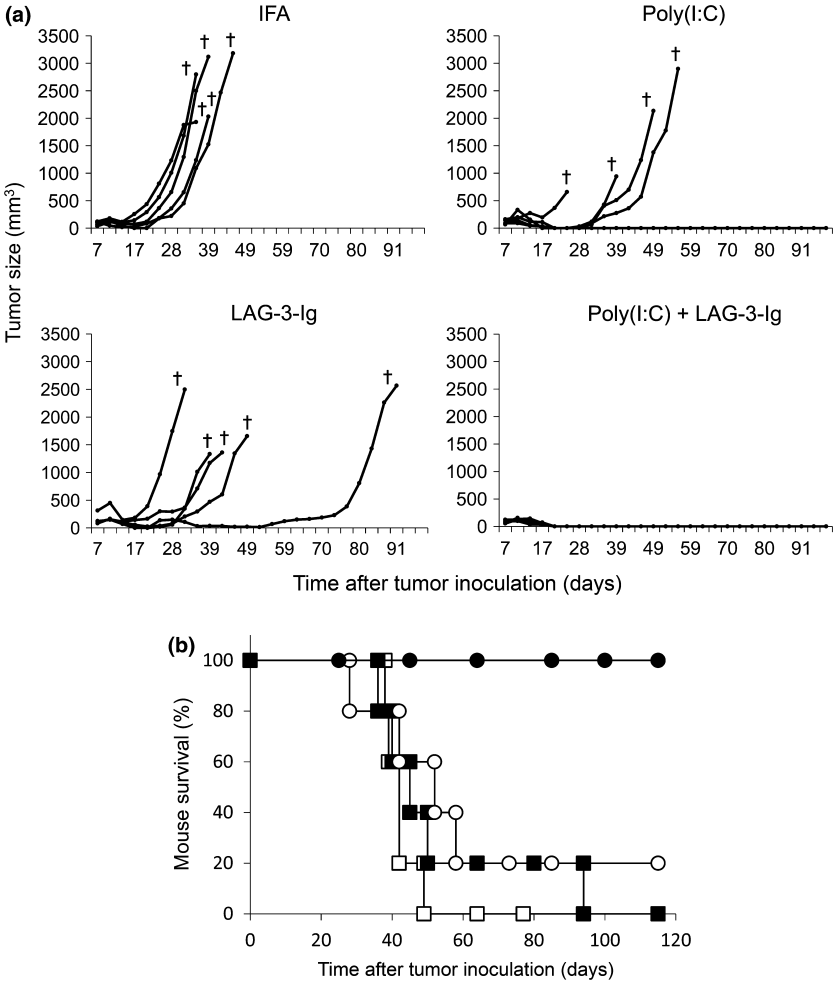

Fig. 1. Eradication of pre-established P815 tumor by P1A peptide vaccine together with combined adjuvant of polyinosinic–polycytidylic acid (poly(I:C)) and lymphocyte activation gene-3 (LAG-3)-Ig. DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific T-cell receptor-transgenic T cells, followed by vaccination of P1A peptide together with incomplete Freund's adjuvant (IFA), poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. (a) Growth of P815 tumor was measured periodically. Each line represents the tumor size of an individual mouse. †Death of mouse. (b) Survival of mice is shown. Each symbol represents distinct adjuvants as follows: □, IFA; ○, poly(I:C); ■, LAG-3-Ig; ●, poly(I:C) plus LAG-3-Ig. The survival of mice treated with poly(I:C) and LAG-3-Ig was significantly longer than the other groups ($P < 0.05$). Representative data of two independent experiments are shown.

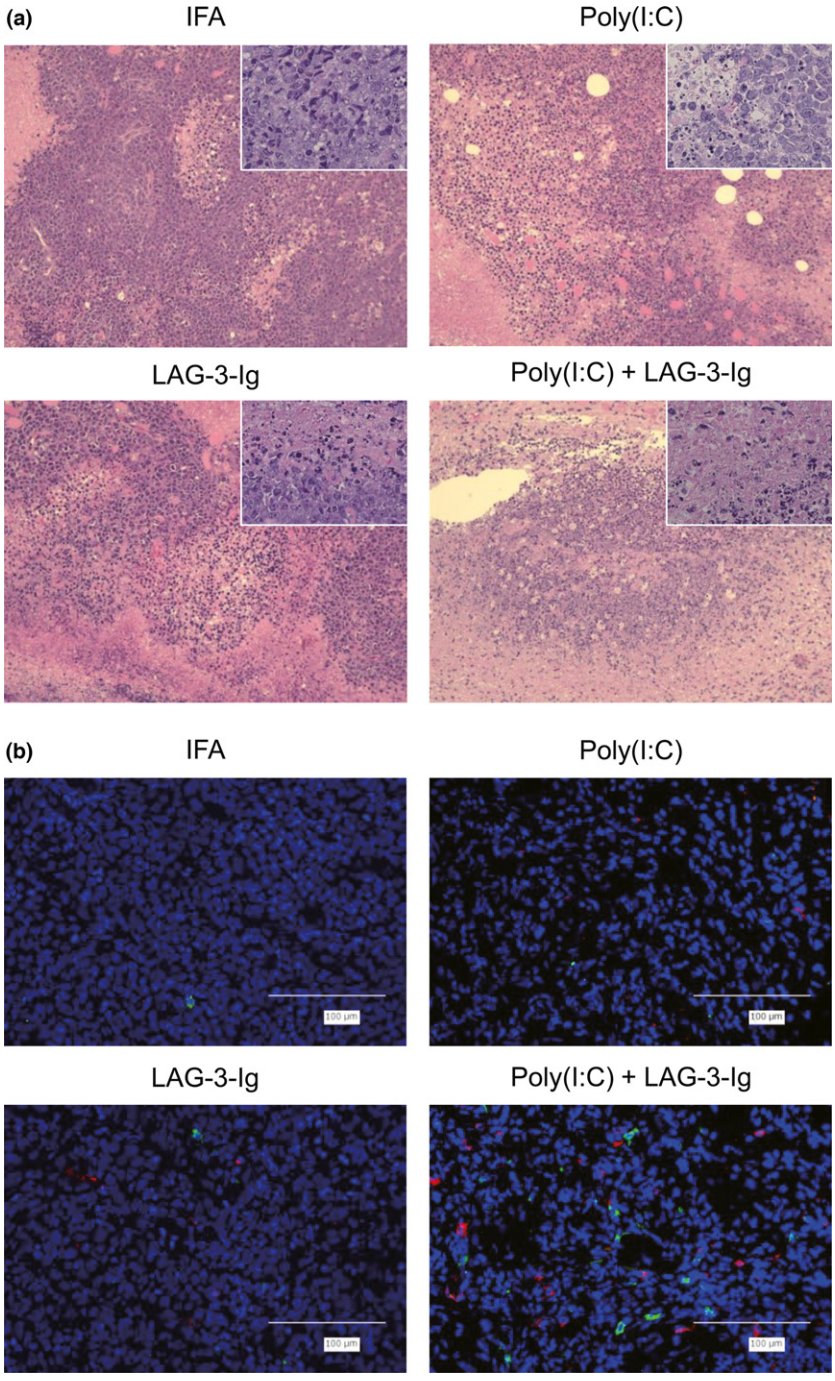

Fig. 2. Histopathological analyses of P815 tumor tissues in mice treated with various adjuvants along with P1A peptide vaccine. DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific T-cell receptor-transgenic T cells, followed by vaccination of P1A peptide together with incomplete Freund's adjuvant (IFA), polyinosinic–polycytidylic acid (poly(I:C)), lymphocyte activation gene-3 (LAG-3)-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumors were surgically resected and subjected to histopathological analyses. (a) Representative images of H&E staining are shown (magnification, ×200). Insets indicate high-power magnification of each image (×400). (b) Representative images of immunohistochemistry staining by anti-CD4 antibody (red), anti-CD8 antibody (green), and DAPI (blue) are shown (×200).

(I:C) and LAG-3-Ig (Fig. S1). These findings confirm that the combined adjuvant of poly(I:C) plus LAG-3-Ig together with peptide vaccine greatly enhances tumor-reactive T cell responses and MHC expression, probably due to IFN-γ produced by infiltrating T cells, in the tumor microenvironment.

Development of tumor-specific memory responses in mice treated with P1A peptide vaccine mixed with poly(I:C) plus LAG-3-Ig adjuvant. DBA/2 mice treated with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig achieved complete eradication of pre-established P815 tumor and survived more than 100 days (Fig. 1). In order to explore the development of P815-specific long-term T cell memory, the mice that had rejected tumor were rechallenged with P815 or L1210, a tumor cell line syngeneic to DBA/2 mice but irrelevant to P815 in terms of antigenicity. The tumor-rejected mice were resistant to the second challenge of P815 but not to the primary challenge of L1210 (Fig. 3). As control, inoculation of the same numbers of P815 and L1210 cells into naïve DBA/2 mice led to outgrowth of both tumors. These results thus indicate that the therapeutic effects of tumor peptide vaccine together with combined poly(I:C) plus LAG-3-Ig adjuvant induces tumor-specific long-term memory responses.

Enhanced proliferation and Th1-type cytokine production in mice treated with P1A peptide vaccine and poly(I:C) plus LAG-3-Ig adjuvant. In order to explore the underlying mechanisms of the antitumor effects induced by poly(I:C) and LAG-3-Ig

adjuvants with peptide vaccine, proliferative activity and cytokine production of tumor-reactive T cells were assessed. The mice were inoculated s.c. with P815 tumor on day 0, injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. On day 21, tumor-regional LN cells were harvested and cocultured with irradiated P815 tumor cells. Proliferative responses of LN cells were significantly enhanced when the mice were treated with combined adjuvant of poly(I:C) plus LAG-3-Ig, compared to either one of them or IFA (Fig. 4a). Similarly, production of IFN-γ and granulocyte/macrophage colony-stimulating factor was increased by the combined adjuvant poly (I:C) plus LAG-3-Ig to levels significantly higher than the other groups (Fig. 4b). In contrast, an increase in IL-4 and IL-5 production was triggered by poly(I:C) and LAG-3, respectively, and the combination of poly(I:C) plus LAG-3 led to only a modest increase compared to poly(I:C) or LAG-3-Ig alone. The production of IL-17 induced by the combination of poly(I:C) plus LAG-3-Ig was lower than that by poly(I:C) alone, but still higher than IFA. These results suggest that efficient antitumor effects mediated by the combined adjuvant poly(I:C) plus LAG-3-Ig were associated with enhanced T cell proliferation and cytokine production, characterized as a preferential upregulation of Th1-type, but not Th2- or Th17-type, responses.

To further confirm the therapeutic effects of combined adjuvants of poly(I:C) plus LAG-3-Ig, we additionally assessed the proliferative activity and IFN-γ production of tumor-reactive T cells without an adoptive transfer of tumor-specific TCR-transgenic T cells. C57BL/6 mice were inoculated s.c. with B16-F10 tumor on day 0, treated with gp100 peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumor-regional LN cells were harvested and cocultured with gp100 peptide. Proliferative responses of LN cells and IFN-γ secretion were significantly enhanced when the mice were treated with combined adjuvants of poly(I:C) plus LAG-3-Ig (Fig. S2). This result confirmed the efficacy of combined adjuvants poly(I:C) plus LAG-3-Ig in a distinct tumor model without an adoptive transfer of tumor-specific T cells.

Prevention of tumor-reactive T cell exhaustion by poly(I:C) plus LAG-3-Ig. It has been reported that tumor-reactive T cells undergo an exhausted status in hosts suffering from progressive tumors.[25] Successful immunotherapies are often associated with prevention and/or reversal of T cell exhaustion.[26] Therefore, we investigated whether injections of poly(I:C) and LAG-3-Ig as adjuvants could influence exhausted phenotypes of tumor-reactive T cells. Mice were inoculated s.c. with P815 tumor on day 0, injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. On day 21, tumor-regional LN cells were harvested and analyzed for the expression of exhaustion markers, including PD-1, LAG-3, TIGIT, and BTLA, on P1A-specific CTLs, which were identified as Vα8.3-positive, CD8-positive cells.[22] It was found that approximately 40–50% of P1A-specific CTLs expressed PD-1 in mice treated with IFA as an adjuvant (Fig. 5). Although treatment with poly(I:C) or LAG-3-Ig as single adjuvants inhibited PD-1 expression to some extent compared to IFA, the combination of poly(I:C) plus LAG-3-Ig showed synergistic effects to remarkably downregulate PD-1 level on P1A-specific CTL. Similarly, expressions of LAG-3 and TIGIT were most strikingly inhibited by the combination of poly(I:C) plus LAG-3-Ig. In contrast, almost no changes in BTLA expression levels were observed by the combined adjuvant of poly(I:C) plus LAG-3-Ig, compared to IFA. As it was reported that CD4-positive, Vα8.3-positive T cells from P1A-TCR transgenic mice also recognize P1A epitope and exert cytotoxic functions against P815 tumor,[27] we further examined exhaustion markers on CD4/Vα8.3 double-positive T cells. Similarly to P1A-specific CTL, expressions of PD-1, LAG-3, and TIGIT, but not BTLA, on CD4-positive P1A-specific T cells were downregulated by the combined adjuvant poly(I:C) plus LAG-3-Ig (Fig. S3). Taken together, these results indicate that antitumor effects induced by peptide vaccine together with the combined adjuvant poly (I:C) plus LAG-3-Ig are associated with prevention of tumor Ag-specific T cell exhaustion.

Discussion

The current study has shown that treatment with the combined adjuvant of poly(I:C) plus LAG-3-Ig profoundly enhances antitumor responses induced by tumor peptide vaccine and leads to complete regression of pre-established tumor in association with long-term immunological memory. Furthermore, mechanistic analyses revealed the activation of Th1-type responses

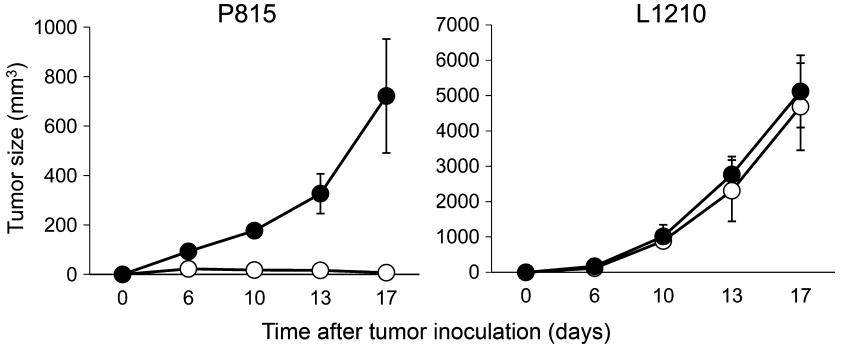

Fig. 3. Induction of P815-specific memory response by treatment with P1A peptide vaccine together with combined adjuvant of polyinosinic–polycytidylic acid (poly(I:C)) and lymphocyte activation gene-3 (LAG-3)-Ig. DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific T-cell receptor-transgenic T cells, followed by vaccination of P1A peptide together with poly(I:C) and LAG-3-Ig on days 8 and 15. More than 100 days later, the tumor-rejected mice (○) were rechallenged s.c. with P815 and L1210 cells at the left and right lateral flank, respectively. As a control, naïve DBA/2 mice (●) were also inoculated s.c. with P815 and L1210 in the same manner. The growth of tumors was measured periodically and is shown as the mean ± SD.

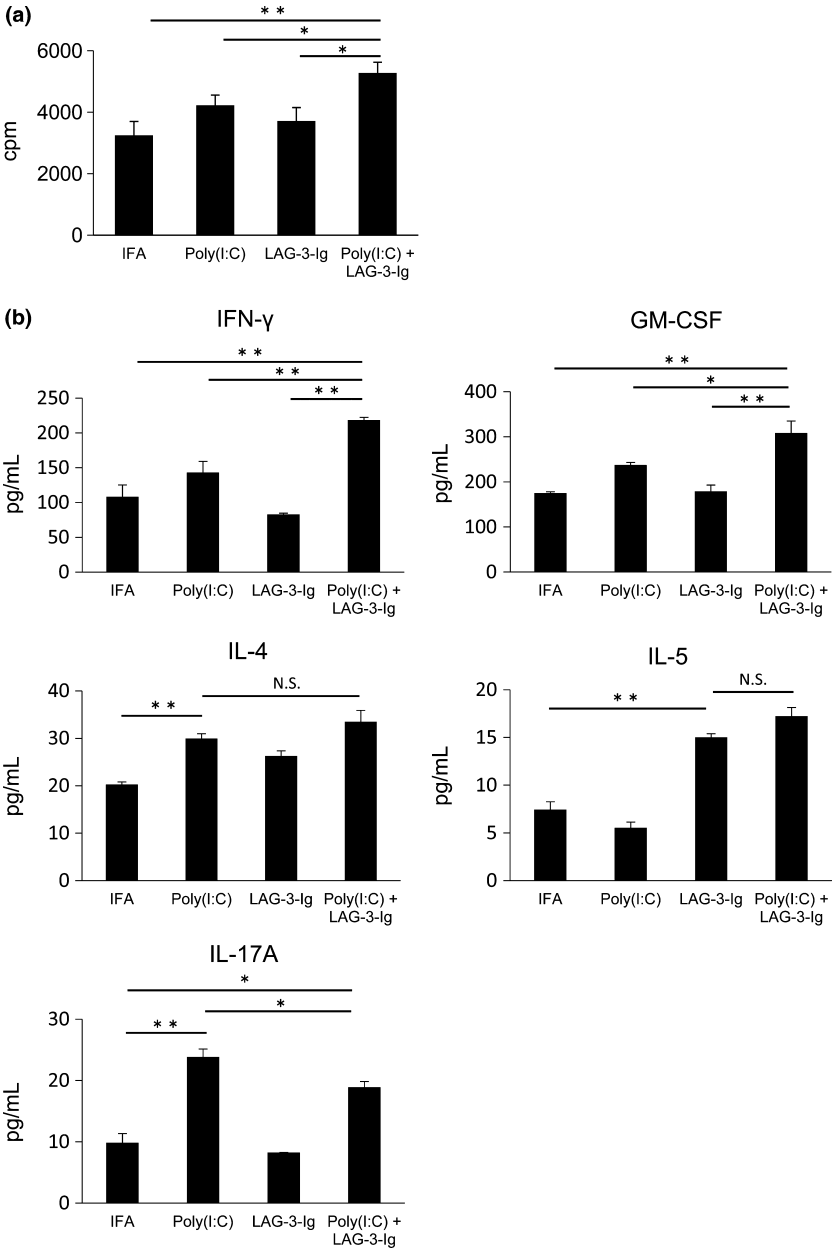

Fig. 4. Proliferation and cytokine production of tumor-draining lymph node (LN) cells in mice treated with P1A peptide vaccine together with combined adjuvant of polyinosinic–polycytidylic acid (poly(I:C)) and lymphocyte activation gene-3 (LAG-3)-Ig. DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific T-cell receptor-transgenic T cells, followed by vaccination of P1A peptide together with incomplete Freund's adjuvant (IFA), poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. On day 21, tumor-draining LN cells were harvested and cultured with 100 Gy-irradiated P815. (a) Proliferative activity of the tumor-draining LN cells during the last 10 h of a 3-day culture was assessed by ^{3}H-thymidine incorporation. (b) After 3 days, the culture supernatants were harvested and the concentrations of cytokines were determined. Representative data of two independent experiments are shown as the mean ± SD of triplicate samples. $*P < 0.05$; $**P < 0.01$. N.S., not significant.

and prevention of the exhausted phenotype in tumor-specific T cells by this treatment. To the best of our knowledge, our study is the first to report a great advantage in combining adjuvants poly(I:C) and LAG-3-Ig for therapeutic cancer vaccine.

Immunologically, there are multiple mechanisms dictating how adjuvants augment antitumor immune responses. Poly(I:C) activates DC to produce type I IFN and IL-12, both of which mediate stimulatory effects on antitumor T cell responses.[28–30] Poly(I:C) also enhances cross-presentation of exogenous Ag by DC, which is necessary for priming CTL specific to TAA epitope in the context of MHC class I.[31,32] In addition, a potential role of poly(I:C) on natural killer cells and tumor cells have been reported.[33,34] However, LAG-3-Ig interferes with inhibitory LAG-3 signal in T cells and prevents them from undergoing exhausted status.[18] In addition, LAG-3-Ig binds MHC class II with a higher affinity than CD4, and induces maturation and activation of DC, which leads to upregulated expressions of CD80, CD83, and CD86.[14,35] Thus, the combination of poly(I:C) and LAG-3-Ig could orchestrate multiple, non-overlapping mechanisms of immune stimulation, which account for profound synergy in therapeutic effects by antitumor vaccine. The fundamental mode of action in poly(I:C) plus LAG-3-Ig adjuvant would be expected to be that DC acquire enhanced APC functions and efficiently present TAA to tumor-specific T cells under the cytokine milieu preferential for Th1-type responses. Subsequently, tumor-specific T cells are activated and efficiently eliminate tumor cells while preventing T cell exhaustion due to resistance to immune inhibitory mechanisms. Lysis of tumor cells is followed by cross-presentation of TAA, which triggers epitope spreading to activate a broad repertoire of tumor-specific T cells, leading to more efficient elimination of tumors.

Recent advances in immune checkpoint blockade therapy, particularly anti-PD-1 Ab, showed remarkable clinical benefits to prolong overall survival and/or progression-free survival in various types of advanced cancers.[36] In this regard, it should

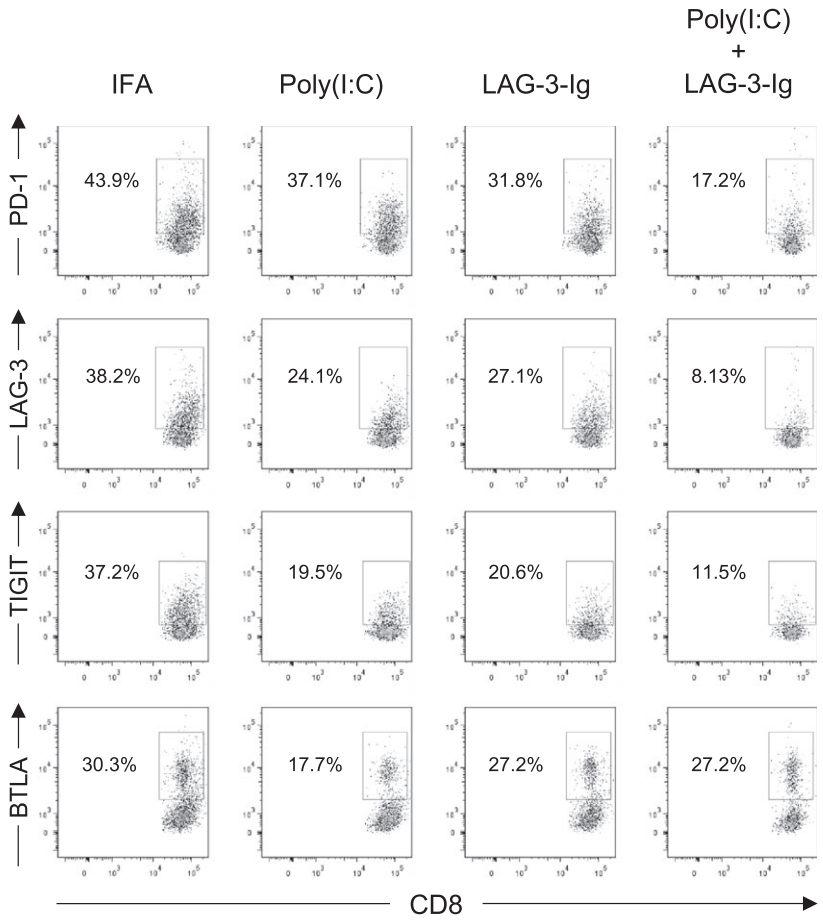

Fig. 5. Expression of exhaustion markers on tumor-reactive CTL in mice treated with P1A peptide vaccine together with combined adjuvant of polyinosinic–polycytidylic acid (poly(I:C)) and lymphocyte activation gene-3 (LAG-3)-Ig. DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific T-cell receptor-transgenic T cells, followed by vaccination of P1A peptide together with incomplete Freund's adjuvant (IFA), poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on days 8 and 15. On day 21, tumor-draining lymph node cells were harvested and analyzed on the expressions of exhaustion markers including programmed cell death-1 (PD-1), LAG-3, T-cell immunoglobulin and ITIM domain (TIGIT), and B- and T-lymphocyte attenuator BTLA, along with CD8 and Vα8.3 by flow cytometer. Expressions of exhaustion markers on P1A-specific T-cell receptor-transgenic CTL, gated as CD8/Vα8.3 double-positive cells, are shown. The numbers indicate percentages of exhaustion marker-positive cells within the P1A-specific CTL population. Representative data of two independent experiments are shown.

be noted that the combined adjuvant of poly(I:C) and LAG-3-Ig is capable of downregulating multiple immune checkpoint molecules on tumor-specific T cells, including PD-1, LAG-3, and TIGIT. It was reported that combined immunotherapies of poly(I:C) with PD-L1/PD-1 blockade in the presence or absence of cancer vaccine induced potent antitumor effects.[37,38] In addition, efficacy of immunotherapies of anti-PD-1 Ab combined with cancer vaccine or other checkpoint blockade, including anti-LAG-3 Ab, has been demonstrated.[39] Thus, downregulation of multiple immune checkpoint molecules by poly(I:C) plus LAG-3-Ig could be a pivotal event accounting for the powerful antitumor effects of this vaccine therapy.

Induction of tumor-specific long-term memory is one of the most important features of cancer immunotherapy, which could protect patients from tumor recurrence. It has been reported that poly(I:C) as an adjuvant of tumor peptide vaccine induces memory CTL responses, in which CD4-positive helper T cells play a supportive role.[40,41] Regarding the effects on $CD4^+$ T cells, our study took advantage of P1A-sepcific TCR transgenic T cells, in which $CD4^+$ T cells are capable of responding to P1A peptide in a MHC class I-restricted manner, as reported by a previous study,[27] and confirmed by our experiment (data not shown). Thus, we consider that the enhanced proliferation and cytokine productions of T cells by the combined adjuvant of poly(I:C) plus LAG-3-Ig might be mediated by $CD4^+$ T cells to some extent; detailed functions of $CD4^+$ T cells in our model need to be elucidated by further experiments. In addition, the LAG-3 signal is known to regulate the quantity of memory T cells *in vivo*.[42] Thus, it is plausible that the combination of poly(I:C) and LAG-3-Ig can induce long-term immune memory and protection from tumor rechallenge, as shown in this study. The LAG-3 signal was also reported to play a crucial role in suppressive functions of Treg.[43] However, the immunological effects of poly(I:C) seem to be irrelevant to Treg numbers and functions.[17,44] In the current study, we could not detect any changes in Treg numbers in mice treated with poly(I:C) plus LAG-3-Ig, compared to other groups (data not shown), suggesting a negligible contribution of Treg to the effects in our approach.

Selection of appropriate and optimized adjuvants is a crucial issue that could govern the clinical success of cancer vaccine therapy. Although IFA has been applied to various clinical trials as a common adjuvant of cancer vaccine, a recent study revealed that TAA-specific T cells are sequestered in the vaccine site and undergo dysfunction and deletion by usage of IFA.[45] Poly(I:C) is currently used in over 20 active clinical trials of cancer vaccine, mainly in glioblastoma, melanoma, and ovarian cancer, by itself or in combination with other adjuvants.[11] Although most of the trials are still early-stage and thus have given no conclusive statements yet, the currently available information indicates the potent immunological effects and promising clinical responses stimulated by poly(I:C).[16,17] LAG-3-Ig has been applied to clinical trials targeting renal cell carcinoma, melanoma, pancreatic cancer, and breast cancer, as an adjuvant of cancer vaccine or in combination with chemotherapy.[18–21] Thus, both poly(I:C) and LAG-3-Ig are readily available as Good Manufacturing Practice-grade

compounds. In addition, their safety profile has been established by multiple phase I clinical trials. Based on the findings in this study, we are planning to implement clinical trials using the combined adjuvant of poly(I:C) plus LAG-3-Ig together with tumor peptide vaccine in the near future.

Acknowledgments

This study was supported in part by a collaborative research grant from NEC Corporation and the Yamaguchi University Pump-Priming Program for Fostering Research Activities.

Disclosure Statement

Tomoya Miyakawa and Shun Doi are employees of NEC Corporation. Shoichi Hazama and Koji Tamada received a collaborative research grant from NEC Corporation.

Abbreviations

Ag	antigen
APC	allophycocyanin
BTLA	B- and T-lymphocyte attenuator
DC	dendritic cells
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin
LAG-3	lymphocyte activation gene-3
LN	lymph node
PD-1	programmed cell death 1
PE	phycoerythrin
poly(I:C)	polyinosinic–polycytidylic acid
TAA	tumor-associated antigen
TCR	T-cell receptor
TIGIT	T-cell immunoglobulin and ITIM domain
TLR	Toll-like receptor
Treg	regulatory T cell

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Expression of MHC class I/II in tumor tissues.

Fig. S2. T cell responses induced by gp100 peptide vaccine with adjuvants.

Fig. S3. Expression of exhaustion markers on tumor-reactive $CD4^+$ T cells.